Attachment B

Introduction

gacA is a member of the SirA gene family. Orthologs of *gacA* exist in *Salmonella serovar typhimurium, E. coli, Vibrio cholera* and *Pseudomonas aeruginosa.* This regulatory protein is believed to control virulence and motility in these species. This protein has been designated a multihost virulence factor in that the protein, GacA, is important for virulence in a wide range of hosts. In *Pseudomonas aeruginosa* this protein has been shown to be important in infections of nematodes, insects, plants and mammals. It is also suspected to play a role in the virulence of *P. aeruginosa* infections in humans.

Our original filing was based on the observation that GacA was shown to be involved in biofilm formation (Parkins et al. 2001). In the paper by Parkins et al. (2001) we showed that a *gacA* mutant formed poor biofilms using the MBEC™ system. Additionally we showed that the *gacA* mutant lost the biofilm phenotype of enhanced antibiotic resistance. To determine if the *gacA* mutant was affected in motility we examined twitching, swimming and swarming and compared this to the parental strain. Unexpectedly we found that twitching and swimming were identical in both the parental and *gacA* mutant strains. In contrast we also showed that the *gacA* mutant was hyper-swarming in comparison to the parental strain. This observation combined with the previous work suggested that *gacA* was a critical factor in controlling virulence, biofilm formation and motility. All these factors likely play critical roles in the development and maintenance of infections. To more tightly establish the link between *gacA* and hyper-swarming we have subsequently gone on to investigate this phenotype.

Electron microscopic studies of *gacA* mutant

We have grown the *gacA* mutant and the parental strain under conditions that allowed the hyper-swarming phenotype. We then examined the strains using an electron microscope. Our findings showed that the parental strain had a single polar flagella. In contrast, the *gacA* mutant had flagella at both poles and two flagella at each pole. Thus, it appears that the *gacA* mutant is hyper-flagellated and this would account for the ability of this strain to hyper-swarm (Figure 1). It should also be noted that in the samples from the *gacA* mutant there was a high degree of sheared flagella present. This might also suggest a high level of expression of the flagella in the *gacA* mutant as compared to the parental strain

Proteomic analysis of *gacA* mutant

The over-expression of flagella in the *gacA* mutant suggested that *gacA* may be a repressor of proteins that control the synthesis of the flagellar machinery. Thus it was of interest to establish if GacA could serve as a repressor or activator of other *P. aeruginosa* proteins. As the majority of proteins involved in the flagellar machinery are either membrane bound or located in the periplasm we have taken a proteomic approach to look for proteins either activated or repressed in the periplasmic fraction of *P. aeruginosa.*

Figure 2 shows that various periplasmic proteins are either activated or repressed in the *gacA* mutant as compared to the parental strain. Some of these proteins have molecular masses and isoelectric points similar to the proteins that make up the flagella biosynthetic machinery. This supports our hypothesis that *gacA* may be repressing the synthesis of the flagella in the parental strain. It also suggests that *gacA* can serve as either an activator or a repressor. This is an important finding as it suggests that *gacA* is indeed a global regulatory gene.

Identification of genes other than GacA involved in the hyper-swarming phenotype.

To add support to our hypothesis that flagellar over-expression is involved in the hyper-swarming phenotype and to determine if other genes may be involved in this phenotype we have undertaken a genetic screen to determine genes that are essential for the hyper-swarming phenotype. To do this we mutagenized the *gacA* mutant with transposon TN5OT152. We then screened for normal swimming and wild type swarming (loss of hyper-swarming phenotype). We screened 3000 colonies and found nine mutants. Figure 3 shows the swarm plates on the mutants. We subsequently cloned the genes mutated in these mutants and were able to get good sequence on four of the clones. The four genes involved were an oxidoreductase, a molecule transporter, a transcarboxylase and the *flgG* gene. The *flgG* gene is of interest because it encodes the flagellar basal body rod protein (see diagram in Figure 4). A mutant in this protein would lack flagella synthesis. We have examined the ability of the mutants to form biofilms. Interestingly, the *flgG* mutant now forms very poor biofilms as compared to the parental strain (Figure 5). This again adds support to our hypothesis that *gacA* regulates flagellar synthesis and flagella play a major role in the hyper-swarming phenotype.

Overall interpretation.

Motility is required for initial contact with a surface in the overall process of biofilm formation. However, as the biofilm matures flagella is not required and it is turned off by the bacteria. Likewise, in biofilm infections such as the lung infections associated with the genetic disease cystic fibrosis flagella synthesis is required for initial colonization but is lost once the infection has become established. Our results suggest that *gacA* inhibits flagellar synthesis during biofilm formation. As such a mutant in *gacA* cannot inhibit flagellar synthesis so the cells have a greater ability to move across a surface and so do not establish a biofilm. Therefore, by targeting the GacA protein we will be able to enhance the swarming of the organism on a surface and block the formation of a biofilm. Clinically, this will likely block the initial stages of the infection and so not allow the bacteria to set up a biofilm and colonize the human host.

Reference:

Parkins,M.D., Ceri,H., and Storey,D.G. 2001. *Pseudomonas aeruginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation. Molecular Microbiology. 40(5): 1215-1226

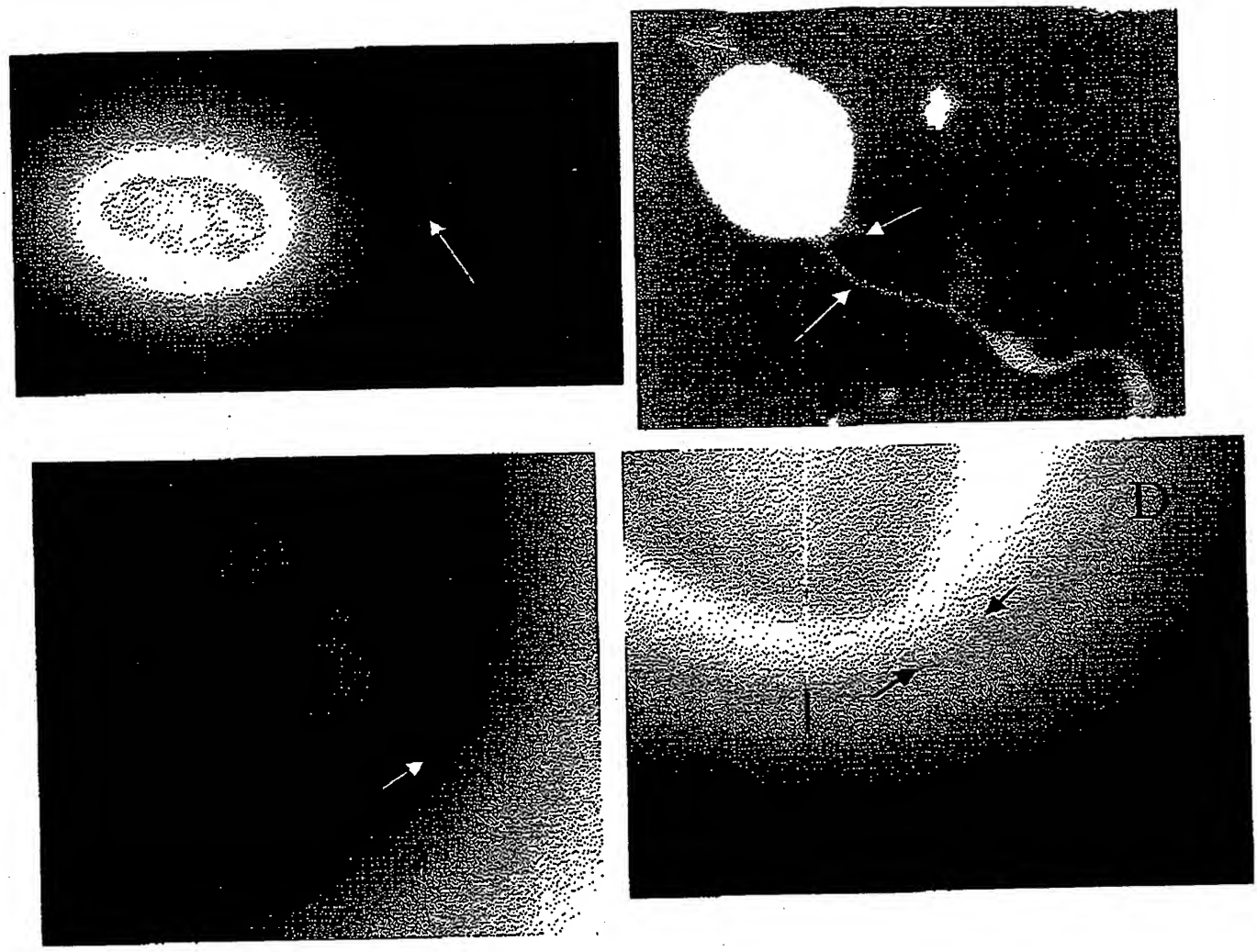
Figure 1. Electron micrographs of *P. aeruginosa* parental strain and the *gacA* mutant. (A) and (C) are parental strains PA14. Note the single polar flagella on these bacterial cells. (B) and (D) are *gacA* mutants. Note the double flagella on the poles of these bacterial cells.

Figure 2. Periplasmic protein extracts of 18hour, planktonic cultures of PA14(a), and PA14*gacA* (b). Total 30 ug of protein loaded per gel (pH3-10NL/12.5% acrylamide /silver-stained) Squares indicate protein spots present in PA14 that are missing PA14*gacA* (GacA activated proteins), whereas circles indicate protein spots present in PA14gacA that are missing in PA14(GacA repressed proteins).

Figure 3. Swarm plates of the nine mutants from a genetic screen of the *gacA* mutant. These are strains that have lost the hyper-swarming phenotype but still swarm in a similar manner as the parental strain. Note the hyper-swarming phenotype of the *gacA* mutant and the lack of the hyper-swarming phenotype in the remaining strains.

Figure 4. Diagram of the flagellar machinery in *P. aeruginosa*. *Note the labelcd protein this is the protein that *flgG* encodes. The rod proteins form the shaft that rotates the hook and flagella of the bacteria. Without this rod the flagella is not anchored and falls out of the bacteria.

Figure 5. Biofilm formation in parental strain PA14 and PA14 *gacAflgG*. The two strains were grown overnight in planktonic culture and then inoculated into two separate MBEC™ devices. Note at all time points tested the level of growth of the *gacAflgG* fusion is below that of the parental strain.



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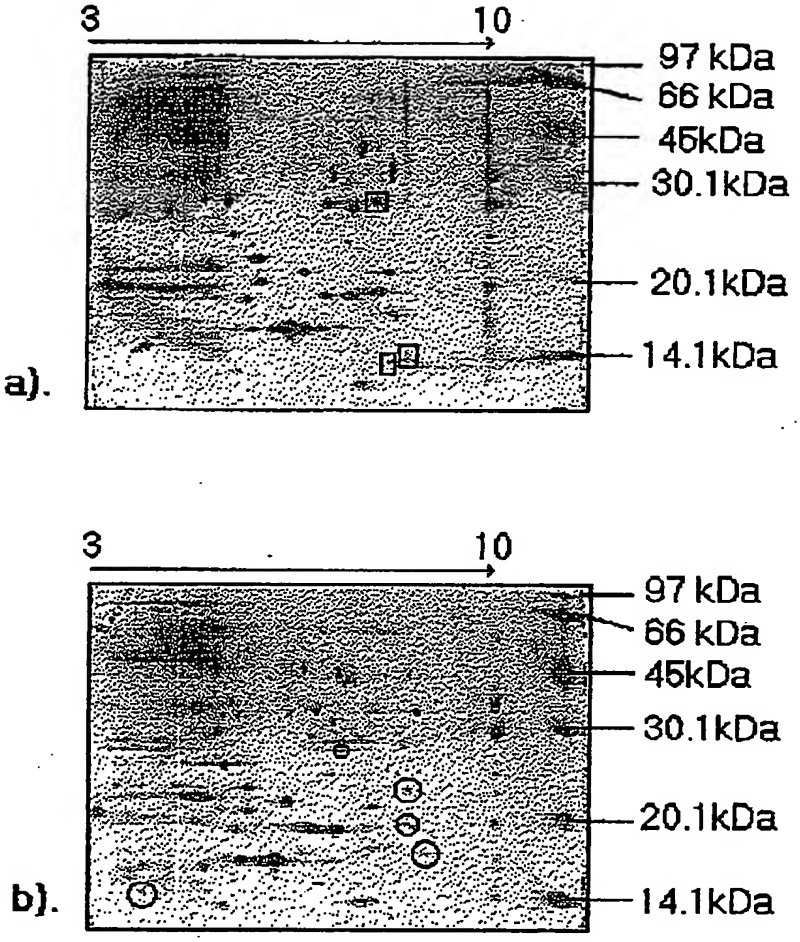

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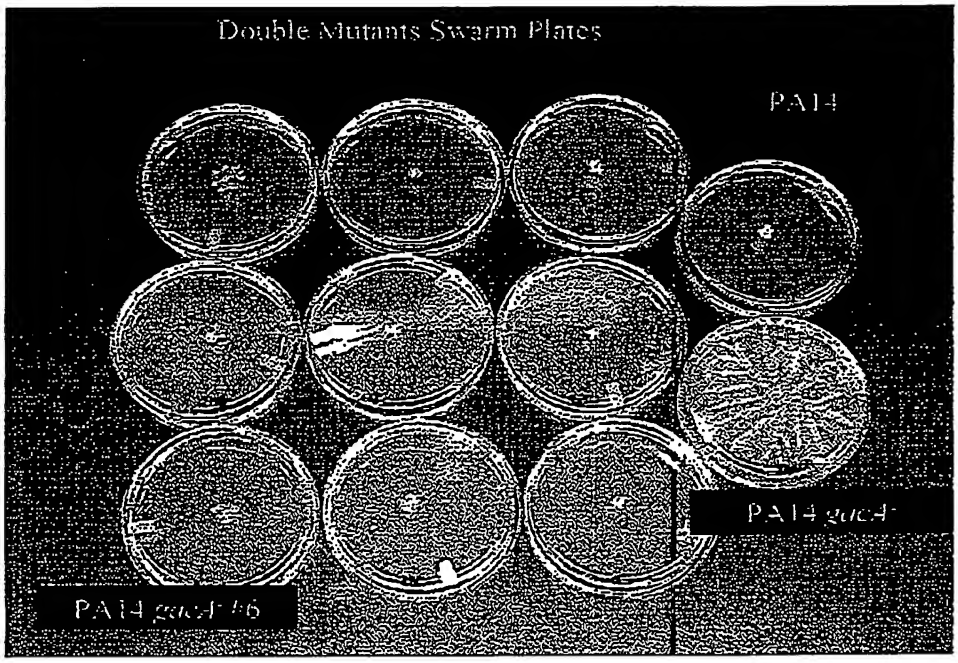

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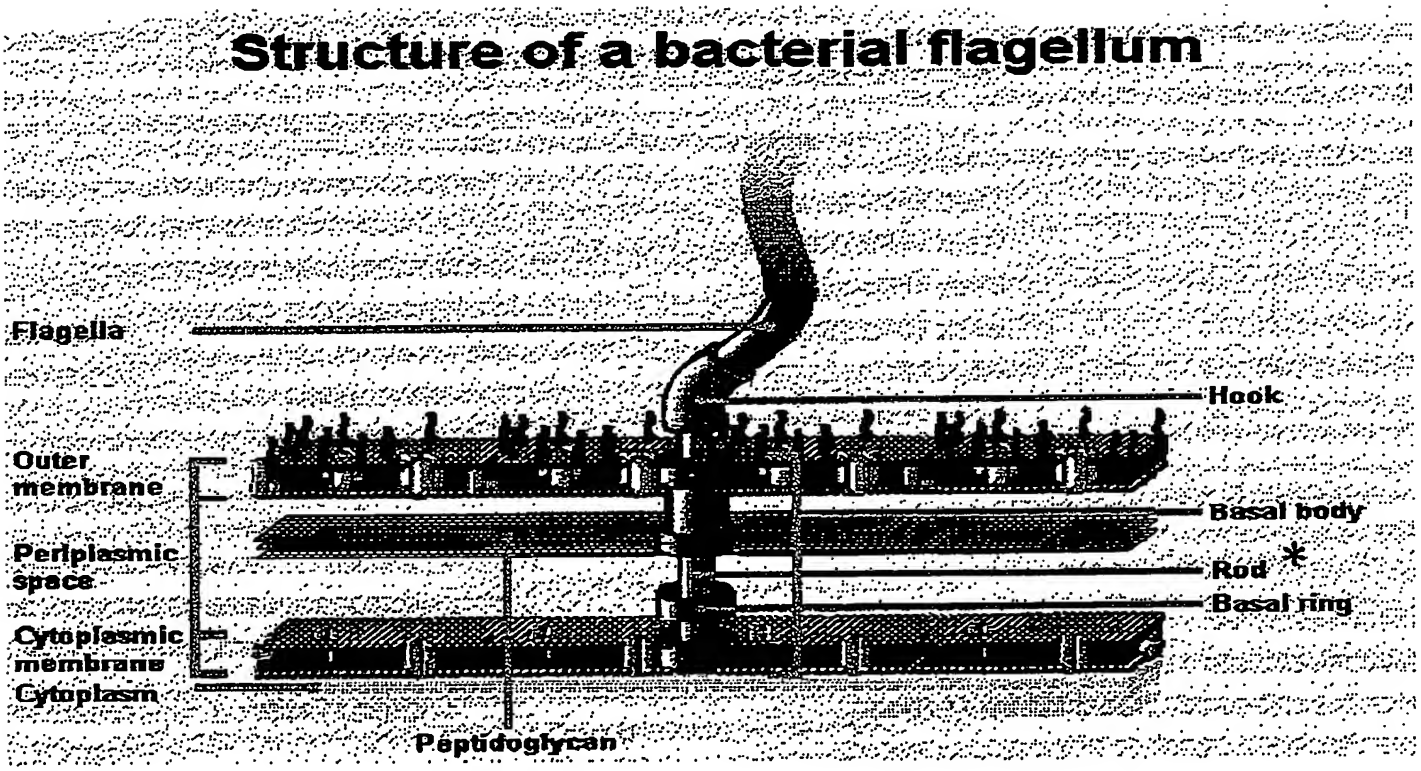

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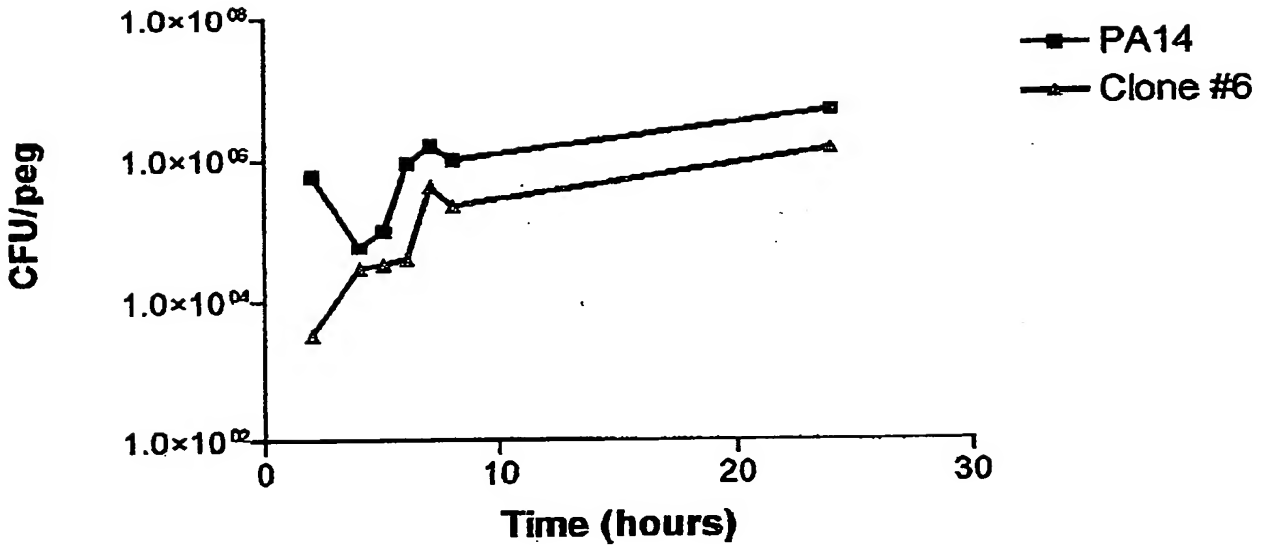

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